Evaluating the efficacy of heart failure drugs on β-adrenergic stress-induced cardiac damage in dystrophic mice.

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in Neuroscience in the undergraduate colleges of The Ohio State University.

By

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Introduction

Duchenne Muscular Dystrophy (DMD) is an X-linked neuromuscular disorder affecting every 1 in 5000 boys (Mendell et al., 2012) and is not inclusive to any one race or culture. While DMD is genetic, one third of cases arise due to new mutations. The underlying pathogenesis of the condition is caused by the absence of the dystrophin protein at skeletal and cardiac muscle sarcolemma. The consequence of dystrophin loss is progressive muscle degeneration and deteriorating muscle force. By age 12, wheelchairs and other equipment for assisted mobility become necessary and the life expectancy for this population is to the second to fourth decade, with an average of 25 years of age (Chadwick et al., 2016; Guiraud et al., 2015).

Dystrophin is associated with a large mechanical complex called the dystrophin-glycoprotein complex (DGC), which is present at striated muscle sarcolemma (Gumerson and Michele, 2011). Through its interaction with this complex, dystrophin provides a mechanical link between the submembraneous actin cytoskeleton and the extracellular matrix (ECM) to provide support to striated muscle membranes during contraction (Gumerson and Michele, 2011). Cardiomyopathy is a major clinical feature in patients with DMD, and typically not visible in early baseline diagnostic examinations. Heart failure resulting from cardiomyopathy is now a leading cause of death in patients with DMD.

There are several theories as to the exact mechanism of cardiomyopathy in this population. One theory is that since dystrophin is associated with the DGC, the mechanical complex needed for a lifetime of normal function of the heart as a pump. Support for this theory comes from observations that restoration of dystrophin in cardiovasculature is not necessary and that a mechanically functional dystrophin in cardiomyocytes is necessary but restoration of the DGC in

cardiomyocytes is not sufficient to prevent the development of a cardiomyopathy in a mouse model of DMD (Hainsey et al., 2003). Signaling through the DGC interacting proteins may also contribute to the phenotype, but without restoration of the mechanical link, there is no prevention of cardiomyopathy.

DMD is characterized by severe muscle degeneration. Skeletal muscle has a regenerative capacity, which eventually runs out and becomes replaced by fibrosis and fat leading to the non-functionality of the muscle. The heart does not regenerate, so degenerated myocytes immediately become replaced by fibrosis. Fibrosis is defined by the excessive unregulated filling of ECM components (Kharraz et al., 2014). It has been seen that the fibrosis can be inflammation-driven. DMD tissue engages in a chronic positive feedback loop of infiltrating mononuclear immune cells for muscle repair but causes myofiber necrosis and muscle injury (Kharraz et al., 2014).

Although gene replacement and correction strategies are under investigation, there is currently no cure for DMD. Therapies that target downstream pathogenic events are likely to improve the longevity and quality of life for patients. As of now, prednisone, a catabolic steroid, is the current standard of care for patients with DMD. Evidence from randomized, controlled trials show steroids improve muscle strength and function in patients with DMD (Angelini and Peterle, 2012). Prednisone fights inflammation in damaged tissues by suppressing the immune system (Birnkrant et al., 2018a; Birnkrant et al., 2018b). While it has significantly improved skeletal muscle function, prednisone has many side effects leading to conflict of whether it helps or hurts the heart. In past preclinical studies with Duchenne muscular dystrophy model mice, compared to untreated mice, steroid treated mice showed increased cardiac muscle

damage and a reduction in heart function and increased pathology (Janssen et al., 2014), suggesting that the long term use of prednisone can have detrimental effects on the heart. Thus, drugs that are effective in targeting myocardial fibrosis and skeletal muscle damage are critical to effective management of this debilitating disease.

Background

The renin-angiotensin system (RAAS) is an important regulatory system of heart and renal function. In RAAS, the liver produces angiotensinogen, and the kidneys produce the enzyme Renin, which cleaves angiotensinogen to angiotensin I. By the action of angiotensin converting enzyme (ACE), angiotensin I is converted to angiotensin II. Angiotensin II plays multiple roles in the body. In the cardiovascular system, angiotensin II causes vasoconstriction leading to increased blood pressure. In the adrenal cortex, angiotensin II leads to the production of aldosterone, which causes NaCl and water retention and increases blood pressure (Eric P. Widmaier, 2014) (figure 1).

The current standard of care for DMD cardiomyopathy is lisinopril, which is given at age 10 (McNally et al., 2015). Lisinopril is a commonly used drug for treating hypertension and heart failure in the United States. The drug acts as an ACE inhibitor, blocking the production of angiotensin II. ACE inhibitors are an effective treatment for high blood pressure and heart failure because they reduce the amount of blood vessel constriction in the body, thereby reducing the amount of work the heart needs to do in order to pump blood throughout the body. Blocking angiotensin II also reduces the formation of aldosterone, a mineralocorticoid

hormone, that is also the last product in RAAS, and works by binding to the mineralocorticoid receptor (MR) (Delyani et al., 2001), therefore, lisinopril is an indirect MR antagonist.

There is also another class of heart failure drugs that has been used in non-DMD cardiomyopathy populations for decades. These drugs directly bind MR to inactivate this receptor which belongs to the steroid hormone receptor transcription factor family. Spironolactone is a nonspecific MR antagonist and eplerenone is a specific MR antagonist. These medications are usually used in late stage heart failure. Since MR is localized in both skeletal muscle and cardiac muscle, these drugs target both locations.

Another relevant receptor is the beta-adrenergic receptor. Beta-adrenergic receptors are located all throughout skeletal and cardiac muscle. The effect of epinephrine (from the adrenal medulla) or norepinephrine (released by sympathetic fibers) on β_1-receptors in heart muscle produces an increase in contractility, having a positive ionotropic effect (Eric P. Widmaier, 2014). Skeletal muscle blood vessels contain β_2-receptors, which mediate vasodilation.

Dobutamine is a beta-receptor agonist and dobutamine stress tests are routinely used clinically in heart failure patients. Dobutamine stress echocardiography is a safe imaging modality in assessing patient heart disease and can identify useful information including high risk features that show a need for surgery (Gentry Iii et al., 2017). These tests are analogous to the stress experienced by exercise on a treadmill but usually are performed on patients who cannot exercise. Previous studies have shown that the dystrophin deficient *mdx* mouse model of DMD is susceptible to dobutamine stress (Meyers and Townsend, 2015). Dobutamine, however, only affects cardiac muscle and has a positive inotropic effect, increasing both the

strength of muscle contraction and force. A very early sign of all heart failure is a dampening of the β-adrenergic response.

Isoproterenol is similar to dobutamine in that it has positive ionotropic effects on cardiac muscle but is an agonist for both β_1 and β_2-adrenergic receptors. Stimulating the β_1-receptor in the myocardium causes an increase in heart rate and cardiac output, and stimulating the β_2-receptors leads to smooth muscle relaxation. β-adrenergic stress tests are used clinically to diagnose cardiovascular disease.

Our lab has used the "het" model of DMD for testing therapeutic strategies to ameliorate disease phenotypes. Het mice are dystrophin-deficient, but also are haploin sufficient for utrophin, a partially compensating paralog of dystrophin. This mouse model shows quantitatively more fibrosis than the dystrophin-deficient *mdx* mouse model, making these mice useful for assessing therapeutic effects on pathology (Zhou et al., 2008). Previous studies in our lab have shown that treating het mice (knockout for dystrophin and heterozygous for knockout of utrophin), with lisinopril and spironolactone starting early in life (4 weeks), there was improved cardiac function, decreased cardiomyocyte damage, and sustained muscle and limb force to 80% of normal levels at 20 weeks of age compared to 40% in the untreated het mice (Rafael-Fortney et al., 2011). To show the effect of early treatment on cardiac outcomes in patients with DMD, a randomized double blind and placebo trial was conducted on boys 7 years or older with a daily oral dose of 24mg of eplerenone, a specific MR antagonist, acting on the RAAS (n=42).

Our group's previous work has shown that since heart abnormalities precede the symptoms, MR antagonists should be used at an earlier time. A clinical trial with the specific MR

antagonist, eplerenone, compared to placebo in patients with DMD showed treatment started at the first detection of myocardial damage slows the progression of cardiomyopathy in DMD (Ramen et al,. 2015; Raman et al., 2017). Preclinical treatment with eplerenone and spironolactone resulted in similar functional and pathological improvements in muscle without changing MR protein levels (Lowe et al., 2016). A second cardiac outcome clinical trial comparing spironolactone with eplerenone in DMD patients is currently underway (NCT02354352).

These drugs are effective because MR has strong selectivity for aldosterone due to the presence of 11β-hydroxysteroid dehydrogenase type 2, 11β-HSD2, located on the cell surface that converts circulating glucocorticoids into metabolites that are unable to bind to MR (Odermatt and Kratschmar, 2012). MR expression has been seen in cardiomyocytes and infiltrating macrophages during inflammation (Odermatt and Kratschmar, 2012). Previous studies in our lab have shown that myeloid immune cells that infiltrate damaged muscle tissue are able to produce aldosterone locally, which can result in over activation of MR that we hypothesize exacerbates damage (Chadwick et al., 2016). By studying levels of aldosterone synthase (CYP11B2), we have also seen for the first time that aldosterone synthesis enzymes are present in muscle-derived leukocytes and levels of CYP11B2 are higher in dystrophic muscle compared to healthy (Chadwick et al., 2016).

Objective

This study aims to determine whether indirect and direct MR antagonists, lisinopril and spironolactone, which are known to improve cardiac function and pathology at baseline in

preclinical models, will prevent against cardiac damage due to isoproterenol induced beta-adrenergic stress in the dystrophin-deficient genotypic *mdx* mouse model of DMD.

Materials and Methods

Mice and preclinical treatment

All experiments were performed under approved IACUC protocol. The animal model used in this study was the *mdx* mouse. They have the same genotype as human DMD with the skeletal muscle degeneration, but do not display severe cardiomyopathy or the clinical signs of DMD and appear grossly phenotypically normal. The isoproterenol stress project included 2 groups of male *mdx* mice, treated with lisinopril + spironolactone (LS) (n=10) and untreated (n=10), one group of LS *mdx* females (n=5) along with 10 male and 5 female (n=15) C57 BL 10 wild type controls.

A second group of mice treated in a follow-up control experiment consisted of the beta-blocker experiment had 2 groups of male *mdx* mice, untreated (n=5) or treated (n=11) with the beta-blocker, metoprolol. All *mdx* mice in this study were bred and in-housed. Mice are weaned at 4 weeks and there were 2-3 mice per cage. LS treated mice were put on the drug treatment at 4 weeks once they are weaned, and stayed on treatment until 21 weeks of age. Mice were treated via water bottles containing drug treatment cocktail; 15 mg spironolactone, the MR antagonist, 8mg lisinopril, the ACE inhibitor, 120 µL of 100% ethanol and 60 mL of reverse osmosis water (approx. 20+37.5 mg/kgxday respectfully). The metoprolol drug treatment consisted of 1 mg of metoprolol in 60 mL of reverse osmosis water (2.5mg/kgxday), treated mice were put on treatment for 2 weeks at 21 weeks of age.

For both treatments, water bottles were replaced every Monday, Wednesday and Friday. All the mice were weighed and the volume of left over drug in the water bottles were recorded every week to ensure the drug is not affecting the taste and consumption of water and confirm approximate dosages.

At the end of the LS treatment, Evans blue dye (EBD) was injected into all of the mice. EBD recipe (10mg/mL) was made with 1 g of Evans blue dye powder in 100 mL of Phosphate buffer saline (PBS), filtered through a 0.2 μm filter. 200 μg/g body weight of EBD is injected in each mouse, therefore the mice were weighed before the injection to insure accurate amount used. EBD was stored in a cool, dry area. Isoproterenol (200 μg/g) was injected 24 hours after EBD injection. The amount injected was determined by the weight of the mouse. Isoproterenol was injected every two hours for a total of three times. All the mice were injected with the isoproterenol stress solution using a 0.5 cc insulin syringe. The needle was inserted intraperitoneally on each mouse. Once each injection had been made, the mouse was observed and any observations were recorded, including potential death of the mouse due to abnormal heart rate (arrhythmia). Two hours after the last injection, mice were euthanized and hearts were removed.

Histology and quantification

After removal, the heart was transversely cut in half. The bottom half of the heart was frozen in liquid nitrogen. The top half was placed in a foil cup, cut side faced down in optimal cutting temperature (OCT) solution, then frozen down in liquid nitrogen cooled isopentane. 8 μm and 30 μm sections of the heart were cut on Cryostat Bright model OTF 5000. 8 μm sections

were cut for immunofluorescence stains, 30 µm sections were cut to better assess the EBD stain. The thicker section allowed for multiple cell layers to be stained for a clearer image of the dye.

One slide per sample was stained with hematoxylin and eosin following standard protocol for assessing overall histology. Other slides are used for Immunoglobulin G (IgG) (Alexa 488 goat anti-mouse IgG, 1:200; Life Technologies) immunofluorescence staining to visualize muscle damage. The immunostain was conducted by first encircling heart sections with a hydrophobic pen, then the slides were immersed in potassium phosphate buffered saline (KPBS) for 5 minutes. The slides were blocked with 1% gelatin in KBPS for 15 minutes then incubated with Alexa 488 goat anti-mouse IgG at 1 to 100 dilution in KPBS + 0.2% gelatin (KPBSG) plus 1% normal goat serum for 1 hour at room temperature in a moist chamber. The antibody solution was poured off and the slides were washed in KPBSG 3 times for 5 minutes. A glass coverslip was applied with vectashield mounting medium containing DAPI to counterstain nuclei.

One slide per sample was used to quantitate the percentage of EBD and IgG of the cross section of the heart to access the amount of cardiac damage present on slides that were coded by a lab member to allow for blinded analysis. To quantitate, overlapping images of the full stained transverse ventricular heart sections were taken under a 10X objective using SPOT RT slider digital camera and SPOT software with a Nikon Eclipse 800 microscope. Images were then composited together on Adobe Photoshop CS6 into a single transverse image of the ventricles. For EBD and IgG stain, damaged areas were painted blue using paint-bucket tool and selected with magic wand, showing the number of pixels selected on the histogram window on right side and the number was recorded. Next, select whole section including the blue areas and record

the number of pixels selected. The percent amount of damage is calculated by [(damage (blue) / total tissue (including blue, excluding white background)] * 100 = percentage of damage.

An analysis of one-way ANOVA was used to determine statistically significant differences in cardiac damage between the C57, LS treated and untreated groups of mice, followed by a Dunnett post hoc to test if there are significant differences between individual groups. A Student's t-test was used to find significant differences between the two beta-blocker groups.

Results

The specific goal of this study was to determine whether lisinopril and spironolactone provided protection from stress induced cardiac damage. A follow up study was done with a beta-blocker, metoprolol, to determine if it decreased stressed induced cardiac damage as well. To visualize the cardiac damage, images were taken of the hearts stained by H&E, IgG and EBD (figure 4). The effects of the drugs were measured by percent damage quantification from immunofluorescent staining of IgG, which is a good indicator of damage by disrupted membranes and accumulation of interstitial fibrosis in dystrophic cardiac tissue and with Evans Blue Dye (figure 2).

From analysis of the IgG stain quantification of the lisinopril and spironolactone group (figure 2), the treated males (n = 10) had an average of 16.9 $\pm$ 3.9 percent of damaged ventricular cross-sectional area. The untreated males (n = 10) had 14.9 $\pm$ 2.5 percent of damaged ventricular cross-sectional area. The control C57s (n = 10) had 3.2 $\pm$ 1.3 percent of damaged ventricular cross-sectional area. The treated females (n = 5) had an average of 22.2 $\pm$ 6.2 percent of damaged ventricular cross-sectional area. The untreated females (n = 5) had 5.5

$\pm$ 2.2 percent of damaged cross-sectional area. And the control females (n = 5) had 4.3 $\pm$ 2.4 percent of cross-sectional damage.

Significant differences were found when using an AVOVA to compare the C57, untreated and LS treated group (p = 0.0002). Followed by a Dunnett test, the significant difference was seen between the C57 group and the untreated group (p = 0.0337). There was no significant difference between the LS treated and untreated groups using a Dunnett test (p = 0.0777).

Separating the groups by sex, there was a significant difference in damage when comparing all the males in the LS study (p = 0.0034). Using a Dunnett test, the C57 group had a significant difference compared to the untreated (p = 0.0118). For the females however, from the ANOVA, there was no significant difference in damage between the C57, treated and untreated (p = 0.3434) (figure 2). This data suggests that the drug combination of ACE inhibitor lisinopril and MR antagonist spironolactone do not protect against beta adrenergic stress in cardiac tissue.

In beta-blocker treatment, the treated *mdx* males (n = 11) had a damaged cross-sectional area of 7.0 $\pm$ 2.9 which was greater than the untreated group (n = 5) that had 2.5 $\pm$ 1.2 percent cross-sectional area damaged. A Student's t-test showed no significant differences between the groups (p= 0.3280).

Discussion

It has been seen that inhibition of MR improves cardiac muscle function, pathology, and damage histology in more severe forms of DMD models. This study aimed to test whether that inhibition also improved the effects of beta-adrenergic stress induced damage from isoproterenol, a beta-receptor agonist, in a mouse milder form of DMD. The damage caused by

the beta-adrenergic agonist was not comparable to damage previously seen by others (Yue et al., 2004). With its high variability, treatment with neither the mineralocorticoid receptor antagonists nor a known beta-blocker was able to demonstrate an effect on decreasing cardiac damage.

In both treatment groups, C57 had significantly less damage, but whether the mice were treated with LS or metoprolol, the treated group had much more damaged cross-sectional area compared to their own untreated groups. Surprisingly, the beta blocker treatment did not have a beneficial effect on the beta adrenergic stress induced damage, the treated groups had a higher percentage of cross sectional area damaged compared to the untreated. Likely due to the highly variable amount of damage from mouse to mouse. Literature has shown ACE inhibitor in combination with beta blockers have shown beneficial effects in DMD patients with heart failure (Ogata et al., 2009). Perhaps in combination, these two drugs will prevent against beta-adrenergic stress. Comparing the amount of cross sectional damage quantitated with IgG and EBD, the hearts labeled with IgG showed more damage and thus it was concluded that IgG is a more sensitive marker than Evans Blue Dye. The study then continued quantifying with only IgG.

This study did not include an *mdx* group without the isoproterenol injection, thus how much damage present before isoproterenol was not known. Previously published data has shown 5% damage in unstressed 20 week mdx (Janssen et al., 2014). Environmental factors such as vivarium caretaker variability, cage transportation, and stressors such as noise or temperature, could have increased baseline damage in this study. There were some practical limitations of

enrolling more mice in this study over a short time period, but in retrospect, these controls should have been included.

Due to the mild cardiac phenotype of the *mdx* mice, which was not as exacerbated as expected, and therefore did not display a dramatic difference from wild-type controls, a better test of the original objective may instead use a more severe model in the future. The het mouse model is also commonly used to study Duchenne muscular dystrophy, this model has a knockout for dystrophin and heterozygous for knockout of utrophin, they are a more severe model compared to *mdx* and would not be best to test for efficacy of this study. Future directions may include testing with the het model. The rationale for not originally using this model was that the beta-adrenergic stress test has never been tested in this *mdx* model and conducting this experiment would require a large amount of testing of dosages and timing.

Immunofluorescence staining for CYP11B2, aldosterone synthase, was conducted on different models of cardiomyopathy to see MR signaling and aldosterone synthesis. However, due to the high background fluorescence in the heart, analysis could not be done accurately. Thus, future experiments are needed to be done to optimize a colorimetric immunostaining to assess the presence of CYP11B2 positive macrophages in dystrophic hearts because it is important to understand the role of CYP11B2 in the heart.

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Figures

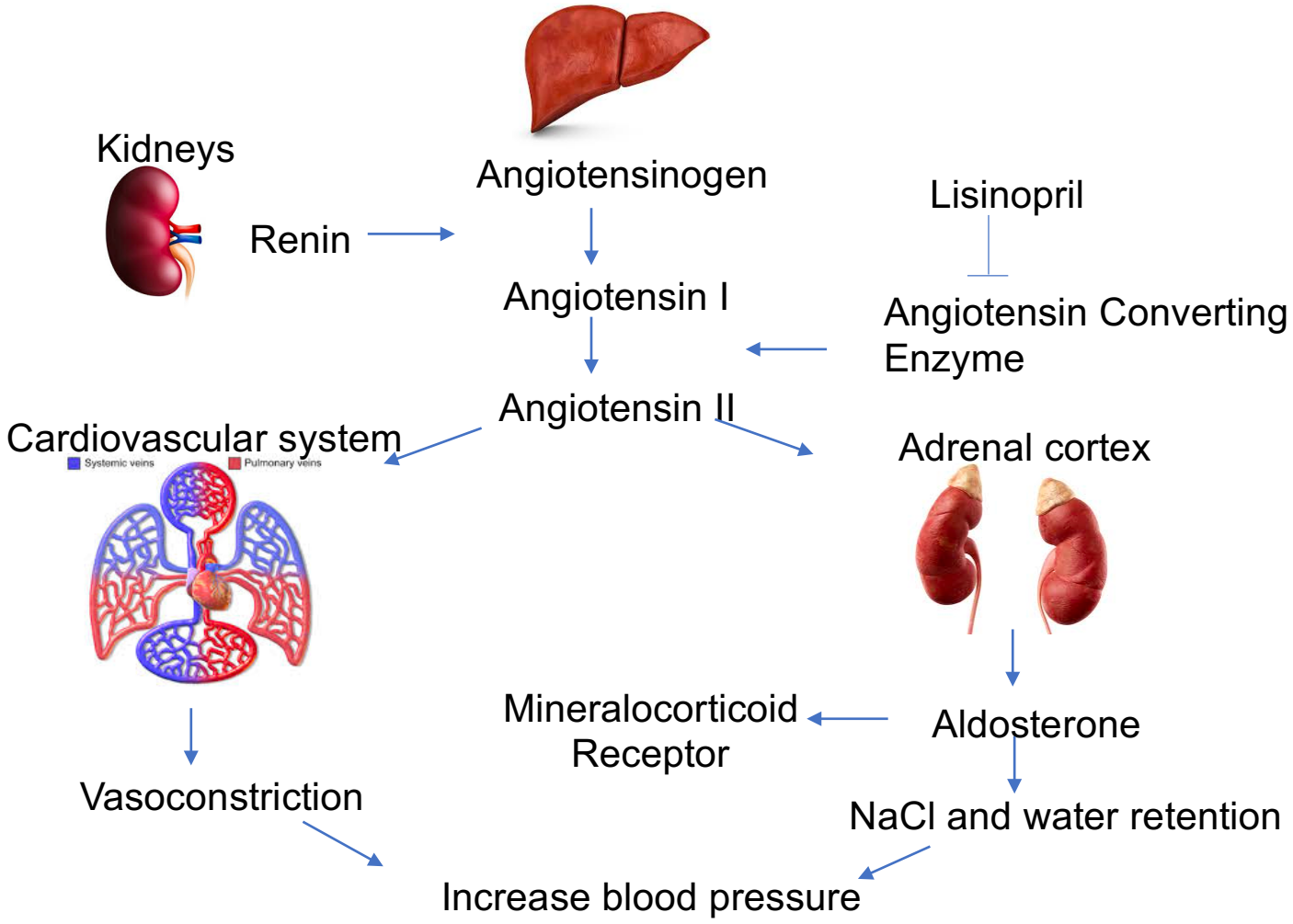

Figure 1.
Figure 1 diagrams the Renin-Angiotensin system (RAAS). In RAAS, the liver produces angiotensinogen and kidneys produce the enzyme Renin, which cleaves angiotensinogen to angiotensin I. By the action of angiotensin converting enzyme (ACE), angiotensin I is converted to angiotensin II. Lisinopril is an angiotensin converting enzyme inhibitor. Angiotensin II plays multiple roles in the body. In the cardiovascular system, angiotensin II causes vasoconstriction leading to increased blood pressure. In the adrenal cortex, angiotensin II leads to the production of aldosterone. Aldosterone is a mineralocorticoid receptor hormone but it also causes NaCl and water retention and increases blood pressure.

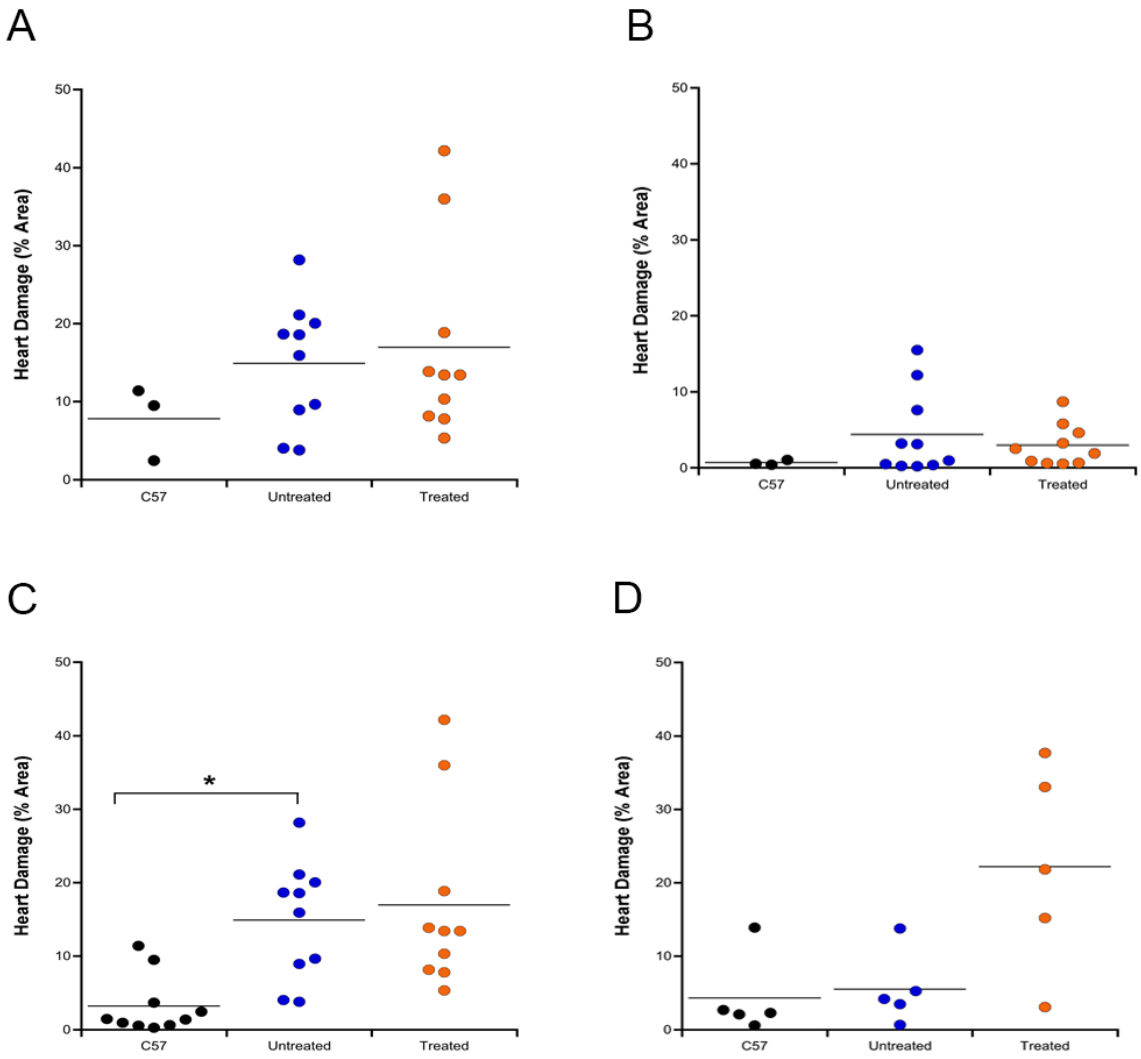

Figure 2.

Figure 2 shows dot plots of data from the lisinopril and spironolactone treatment. Graphs A and B show the percent of cross-sectional area (CSA) heart damage from the same set of mice stained with Immunoglobulin G (IgG) and Evans Blue Dye (EBD) staining. A shows the percentage of IgG heart damage in C57 wild-type controls, and treated and untreated *mdx* dystrophic male mice as quantified from IgG staining. B shows the percentage of heart damage in C57 wild-type controls, and treated and untreated *mdx* dystrophic male mice as quantified from EBD staining. IgG is a more sensitive and accurate marker for damage compared to EBD. Graphs C and D show only IgG quantified heart damage from the *mdx* mice on the LS treatment. C shows the percent of CSA of male *mdx* mice. The C57 control group had a significant difference of CSA damage compared to the untreated group ($p = 0.0118$). Graph D shows IgG quantified heart damage from the female *mdx* mice. $p < 0.05$ indicated by *.

A

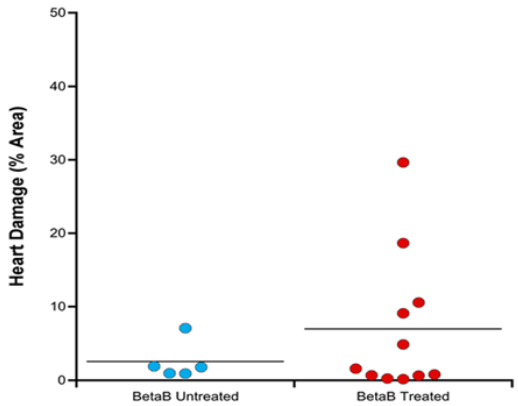

Figure 3.
Figure 3 shows percent of cross-sectional area damage quantified by IgG of male untreated and treated *mdx* mice from the beta-blocker treatment. There were no significant differences between the treated and untreated groups.

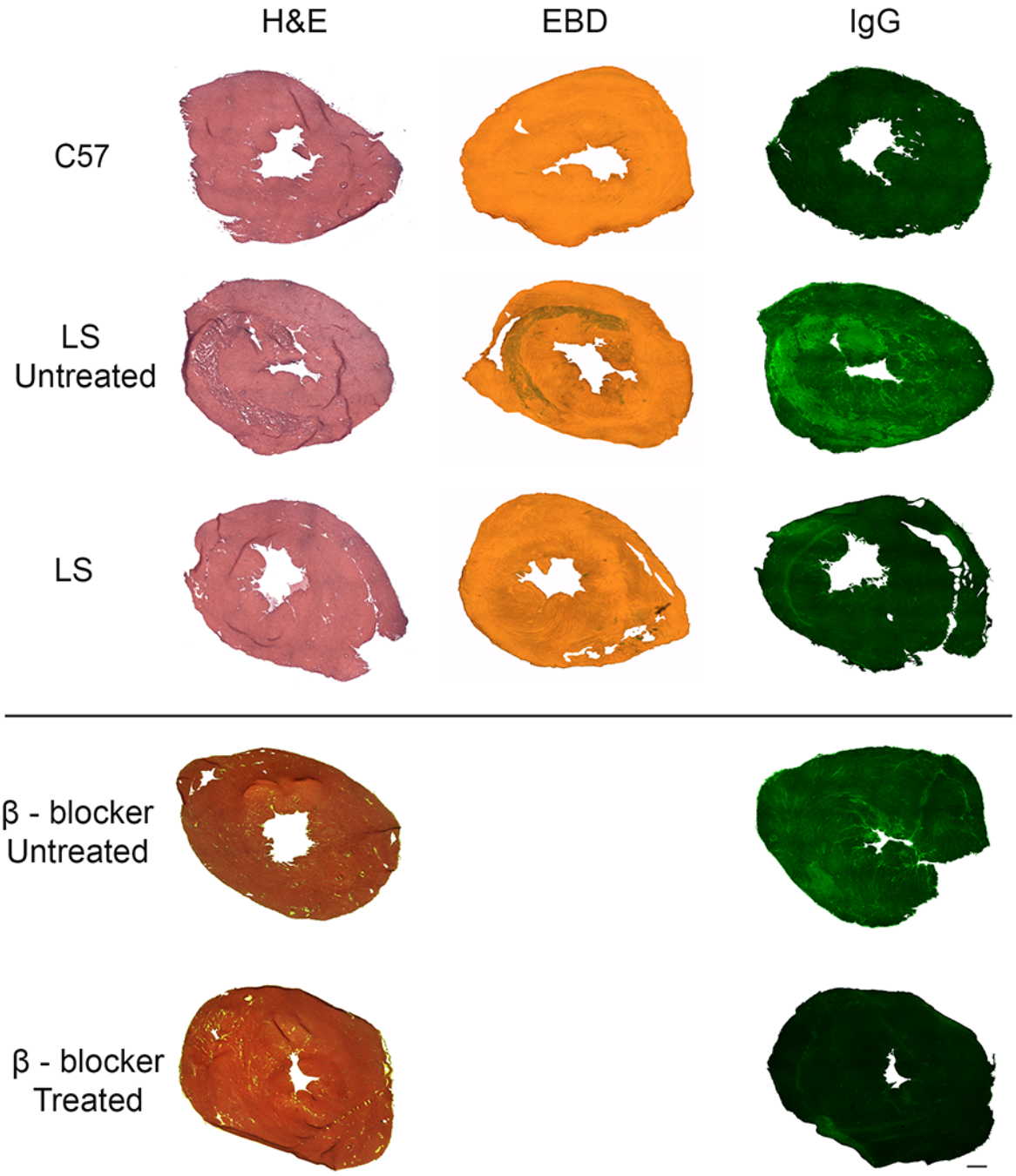

Figure 4.
Figure 4 shows composite micrographs of immunofluorescence stain of Evans Blue Dye (EBD), Immunoglobulin G (IgG), and Hematoxylin and Eosin (H&E). The top is of LS treated, untreated, and C57 *mdx* hearts. The bottom shows composite micrographs of immunofluorescence stain of H&E and IgG of beta blocker treated and untreated *mdx* hearts. Due to IgG being a more sensitive marker for damage, EBD composites and quantifications were not done for the beta-blocker mice. Bar = 400μm.